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Studies on the Extraction of Parathyroid Hormone from Urine

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STUDIES ON THE EXTRACTION OF PARATHYROID HORMONE FROM URINE

by

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LIFE

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In September of 1963, he began his graduate studies in Pharmacology at Duquesne University School of Pharmacy. From September, 1963, to June, 1964, he served as a graduate teaching assistant.

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CHAPTER I

INTRODUCTION

The possibility that the hormone of the parathyroid glands (PTH) might be present in mammalian urine was first suggested by the finding of considerable amounts of radioactivity in the dialysed urine of mice injected with parathyroid extract (Lilly) labeled with iodine-131 (15). Since the commercially available hormone preparation is a heterogenous mixture of proteins, this radioactivity could have been contributed by a biologically inactive protein. However, since the hormone forms a considerable part of the commercial preparation, it is likely that the radioactivity recovered in the dialysed urine would have been contributed at least in part by the labeled hormone itself or a labeled degradation product.

Employing a bioassay based on the renal action of PTH, namely, the increase in urinary excretion of inorganic phosphate in mice, Davies (6) was the first to show that the urine of patients with hyperparathyroidism contained increased amounts of a PTH-like material. Values of 103-146 U. S. P. units of parathyroid hormone activity were reported for twenty-four hour urine specimens. The range for nomal subjects was reported to be 47-72 units/24 hours whereas in cases of hypoparathyroidism,

the amount was too small to be detected. The assay was insensitive to 24 hour urine extracts containing less than 30 units.

The extraction of the PTH-like material from the urine was carried out by using benzoic acid. Benzoic acid was chosen as an adsorbent because it had been used successfully in the preparation of PTH from ox gland extracts (13). It was found that injection of 1 ml of a saturated solution of benzoic acid in 0.9% saline had no effect on urinary phosphate excretion, nor did it prevent the response to the injected commercial product. This eliminated the likelihood that the main non-protein contaminant (benzoic acid) had an effect on urinary phosphate levels. Extracts of urine from hypoparathyroid patients possessed no detectable parathyroid hormone activity. Therefore, it is unlikely that other urinary proteins were influencing the phosphate response in this assay. The phosphaturic activity of the benzoic acid extract prepared from normal urine was not lost on boiling for 10 minutes at pH 3.5; neither was that prepared from the commercial product. This stability of the extract under acidic conditions provided evidence for the PTH-like character of this extract. Collip (5) prepared PTH by boiling ox glands with an equal volume of 5% HCl for one hour. Davies concluded, therefore, that the preparation obtained from human urine may actually contain PTH. On the other hand, the hormonal principle found in urine may be a metabolite of the original

PTH molecule, still containing that residue essential for biological activity.

Using a bioassay based upon the phosphaturic response to PTH in rats, Fujita et al. (9) showed by using a modified method of Davies that normal urine contained a PTH-like material. In three out of five normal subjects, they reported that the amount of PTH activity in a 24 hour urine sample ranged between 10-30 U. S. P. units, while in the others it was undetectable. Employing the Lilly commercial preparation as a working standard, they reported hormonal recovery values of 75%, 48% and 51%. The values reported by Davies (6) were somewhat higher, viz. 75% and 86%.

Aware that there are other benzoic acid adsorbable hormones in the urine, Fujita et al. tested for a possible influence of some of these hormones. The results with ACTH, triiodotyrosine chorionic gonadotrophin were found to be similar to those obtained with injections of 0.9% saline.

Although no experimental data were given, Kleeman (10) reported that he was able to extract material from human urine, using the method of Davies, which exhibited calcium mobilizing activity when injected into thyroparathyroidectomized (TPTX) rats. He reported that his recoveries were erratic and that cysteine appeared to enhance the chances of recovering this material from the urine.

More recently, the urinary excretion of PTH in man has been investigated with the use of the Munson assay procedure which is based on a hypercalcemic response (12). Eliel et al. (7) determined that in seven out of eight human subjects the PTH-like activity excreted in the urine ranged from 20 to 37 units/24 hours. Six of the patients were presumed normal and two were hospital patients without bone disease or abnormalities of calcium metabolism. One normal had undetectable activity for unexplained reasons. In three hyperparathyroid patients, values of 59, 104 and 120 units/day of parathyroid hormone activity were recorded. Four patients who developed hypoparathyroidism following thyroidectomy had no demonstrable hormone activity in the urine. Upon treatment by intramuscular administration of the commercial extract, one patient who received 400 units/day excreted 73 units/day or approximately 18% of the administered dose whereas a second patient receiving 200 units/day, excreted 37 units/day (19%). The authors offer no explanation as to the chemical nature of the material extracted from human urine. It appears that their bioassay for urinary parathyroid activity is reliable and the results coincide with clinically recognizable differences in parathyroid function.

STATEMENT OF THE PROBLEM

Thus far, no work has been done to elucidate the nature of the PTH-like material contained in the urine. That such material

exhibits PTH "activity" has been shown.

In this research, an attempt has been made to determine the character of this PTH-like material in the hope of shedding some light on the matter of whether this active principle is PTH or a metabolite thereof. Several distinct procedures were employed in an attempt to obtain the hypercalcemic principle from urine. Firstly, a benzoic acid extraction procedure was employed utilizing the method of Eliel et al. (7). Secondly, Sephadex (Pharmacia Fine Chemical) was used to remove the active principle from urine and thereby establish its relative molecular size. Thirdly, it was reported by Rosselin et al. (14) that microfine granules of precipitated silica could adsorb ^{131}I-PTH (iodine-131-PTH) from human plasma. This method, applied with slight modification, involved a series of centrifugations and finally lyophilization to obtain the test material. Aurbach (3) was able to isolate PTH from living tissue by extraction with 70% phenol solution. This method was also employed with slight modification to try and obtain PTH from urine.

Since urine from hyperparathyroid human patients was not available, hyperparathyroidism was induced in rats by injection of commercially available parathyroid extract. The rat urine was collected in these cases and the procedures noted above were applied to obtain the PTH-like material. The hypercalcemic activity of this test material was determined by the Munson bio-

assay method.

CHAPTER II

MATERIALS AND METHODS

A. BENZOIC ACID EXTRACTION OF PARATHYROID HORMONE

The separation of hormone activity from urine was carried out by a modification of the method of Eliel *et al.* (9), which depends on precipitation of urinary polypeptides by benzoic acid and removal of the acid by ethanol.

Male Holtzman rats (130-150 grams) were placed in metabolic cages and given powdered Rockland Laboratory Animal Diet and tap water. Each animal was injected with 200 U. S. P. units of commercial parathyroid extract (Lilly) on each of two days. Six animals were used and the urine was collected over a 24 hour period and refrigerated. Fresh bags (Whirl-Pak bags, Scientific Products, Chicago) were used to collect each 24 hour urine sample. The urine collected over a 48 hour period was pooled and the final volume was approximately 100 ml. The urine was then filtered to remove large particles of food and other debris.

The urine was then acidified with 3 N HCl to pH 3.5. This was then filtered in the cold with Whatman No. 1 filter paper. To this filtrate was added at a slow rate, with constant stirring, one volume of a saturated solution of benzoic acid in absolute ethanol (90 g in 300 ml) to each 10 volumes of urine. Stirring

was continued for a minimum of six hours in the cold. The suspension was then filtered through Whatman No. 54 filter paper on a Büchner funnel, and dried overnight under vacuum in a desiccator with sodium hydroxide pellets. The precipitate was redissolved in 200 ml of absolute ethanol, and then transferred to two 100 ml pyrex glass centrifuge tubes and centrifuged at 1000 rpm in a Model PR-2 International Refrigerated Centrifuge at 0°C for 15 minutes. The wash procedure with ethanol was repeated twice with 100 ml of absolute ethanol being utilized each time. The precipitate was then transferred to two 50 ml polycarbonate tubes to each of which was added 30 ml of absolute ethanol. The tubes were capped and centrifuged for 30 minutes in the cold at 8000 rpm in a Servall SS-1 Centrifuge. The supernatant was discarded and the precipitate was transferred to a watch glass and dried overnight under vacuum in a desiccator.

A known volume of physiological saline (0.9%) was added to the precipitate and this suspension was centrifuged at 8000 rpm for 30 minutes. The supernatant obtained comprised the test material.

B. EXTRACTION OF PARATHYROID HORMONE WITH SEPHADEX

To determine the molecular size of the active principle, various grades of Sephadex (Pharmacia Fine Chemicals) were employed. Sephadex particles have the ability to concentrate

liquids and, depending upon what grade i. e., G-10, G-15, etc., is used, exclude solute molecules of a particular molecular size. Upon centrifugation of this Sephadex in special tubes, a small fraction of the original volume will be obtained containing the excluded solute molecules. This remaining liquid fraction was then lyophilized and tested biologically using the Munson method (12).

Urine was collected from hyperparathyroid rats over a 24 hour period in metabolic cages. Induction of hyperparathyroidism and collection of urine were accomplished as noted above. The collected urine was filtered to remove debris and then acidified to pH 3.5. Depending on its water regain value (expressed as gram H_2O/gram of dry Sephadex), a known amount of dry Sephadex of the desired grade was added to concentrate the urine to a final volume of approximately 25 ml. The Sephadex was allowed to swell for 20-30 minutes in the cold and then it was packed into Filtefuge tubes (Occomy Associates, Chicago) and centrifuged at 2000 rpm for 10 minutes. The urine collected after centrifugation was then lyophilized. The dry precipitate was taken up with a small amount of dilute acetic acid and placed in a freezer to prevent deterioration. Prior to the bioassay, the sample was removed and allowed to thaw. A known volume of physiological saline was added and the mixture was centrifuged at 1000 rpm for 10 minutes to remove insoluble components. The supernatant ob-

tained comprised the test material.

C. EXTRACTION OF PARATHYROID HORMONE WITH SILICA GEL

Samples of urine were acidified and filtered with Whatman No. 1 filter paper. To the filtrate was added a specified quantity of silica gel particles ("Quso G32", Philadelphia Quartz Company, Philadelphia). This suspension was centrifuged and the supernatant was discarded. The precipitate was taken up with doubly-distilled water and recentrifuged. Again, the supernatant was discarded and the precipitate was mixed with an acetic acid-acetone mixture and recentrifuged. The precipitate was discarded and the acetone was removed from the supernatant. The supernatant was then lyophilized, dissolved in a mixture of 0.2% acetic acid and 0.9% saline, and tested biologically.

D. EXTRACTION OF PARATHYROID HORMONE WITH AN ACETIC ACID: ACETONE MIXTURE AND ETHER

In Aurbach's (3) procedure, phenol was used to extract PTH from tissue because it has the ability to extract protein in the presence of nucleic acids, and it stabilizes a solution containing PTH. In the recovery experiments attempted here with commercial extract and urine, phenol was already in the medium and was, therefore, not employed in the experimental procedure.

To an acidified sample of human urine was added a specified amount of PTH. A solution of acetic acid-acetone, 1:4, and 0.004

M in sodium chloride was then added, and after standing for a period of time in the cold, the mixture was centrifuged. In one case, the supernatant was discarded and the precipitate was dried, taken up with 0.2% acetic acid and 0.9% saline, and tested biologically. In another experiment, the precipitate was discarded and to the supernatant was added ether. The mixture was kept overnight in the cold in hopes that the active principle would precipitate out of solution. As will be pointed out later, this was not the case.

E. THE BIOASSAY

An assay similar to that of Munson (12) was employed. Male Holtzman rats (130-150 g) were placed on a calcium-deficient diet (Nutrional Biochemical Corporation, Cleveland, Ohio) and distilled water for three days. On the fourth day, the animals were thyroparathyroidectomized. After the operation, the animals were replaced on the diet and distilled water regimen. On the following day, a tail blood sample was taken. The rats were then anesthetized with ether and various doses of a particular test medium were administered subcutaneously. The animals were then returned to their cages and six hours later were bled again. The serum was measured according to the method of Ashby and Roberts (2) before and after the injection of the test material, and the difference in serum calcium was used to evaluate the hypercal-

cemic activity of the test material.

F. DOSE-RESPONSE CURVES

1. Rats on a Calcium Deficient Diet

Male Holtzman rats (130-150 g) maintained on a calcium deficient diet and distilled water for three days were thyroparathyroidectomized. Twenty-four hours later, the animals were bled from the tail. They were then anesthetized with ether and given doses of 15, 30, 40 and 50 U. S. P. units of the commercial extract (Lilly) subcutaneously. Six hours later, a second blood sample was taken. The rise in serum calcium over the six hour period for each animal was determined and the data are given in Table I. The relationship between the average rise in serum calcium and the logarithm of the dose is shown in Figure 1.

2. Rats on a Normal Diet

Male Holtzman rats were maintained on a normal diet (Rockland) and tap water. They were then thyroparathyroidectomized and 24 hours later, the same procedure was followed as with the animals on a calcium deficient diet except for a difference in dose administration. These animals were given 10, 20, 30 and 40 U. S. P. units of PTE per 100 g of body weight (Figure 2). The rise in serum calcium was plotted against the dose in U. S. P. units/100 g. The serum calcium data are given in Table II.

TABLE I

DATA FOR THE LOG-DOSE RESPONSE CURVE FOR ANIMALS ON A CALCIUM DEFICIENT DIET

Serum Calcium

Rat #	Dose (U. S. P. units)	Before Injection mg%	After Injection mg%	Change mg%	Mean ± Standard Error
1	15	9.43	12.76	3.33	
2	15	7.72	10.39	3.67	
3	15	8.37	12.34	3.97	3.8 ± 0.21
4	15	8.48	13.06	4.57	
5	15	10.92	14.50	3.48	
6	30	10.36	14.70	4.34	
7	30	10.99	15.26	4.27	
8	30	10.21	14.90	4.69	4.7 ± 0.35
9	30	8.36	14.42	6.06	
10	30	8.52	12.71	4.19	
11	40	10.92	16.31	6.39	
12	40	10.91	14.94	4.03	
13	40	8.35	14.65	6.30	5.1 ± 0.52
14	40	8.73	13.43	4.70	
15	40	10.04	13.99	3.95	
16	50	10.92	17.83	6.91	
17	50	10.48	15.29	4.81	
18	50	9.81	15.80	5.99	5.3 ± 0.58
19	50	9.73	14.71	4.98	
20	50	10.60	14.31	3.71	

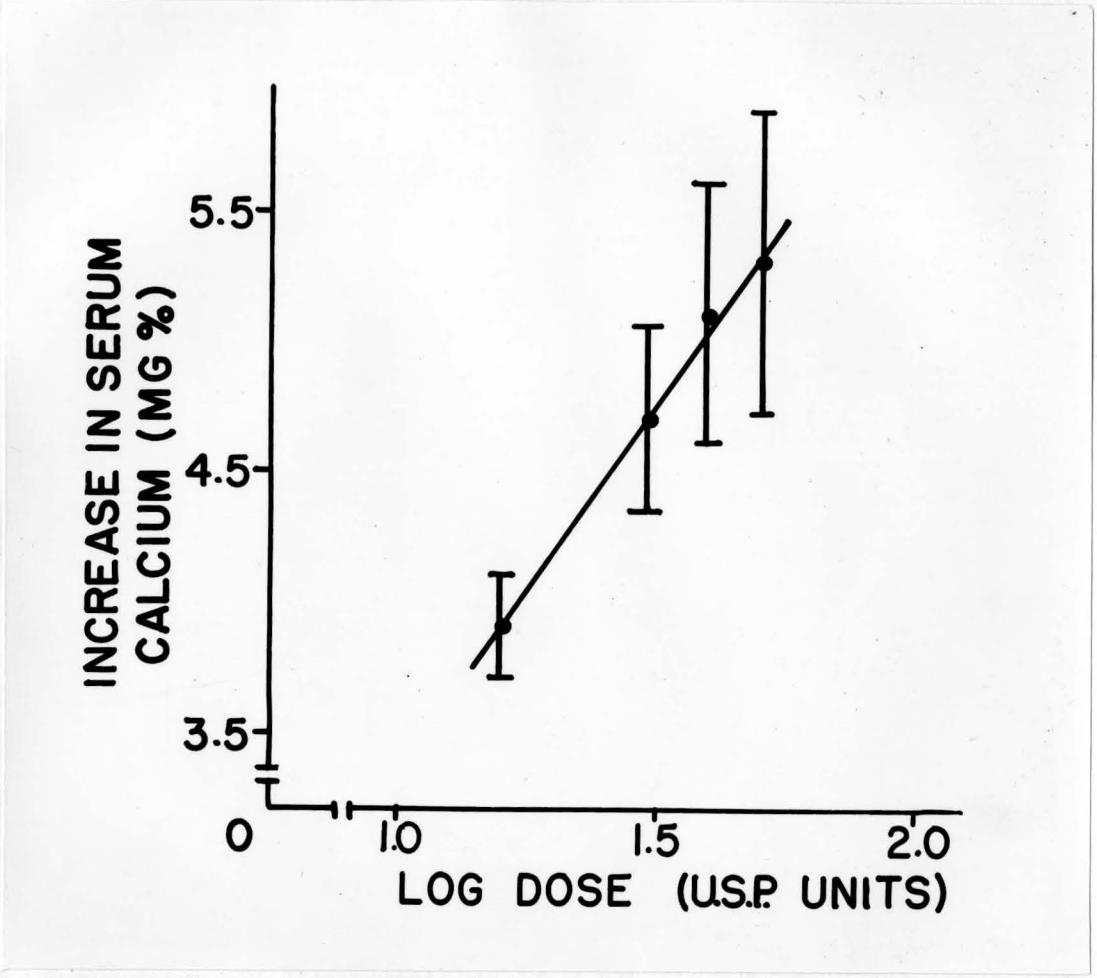

Figure 1

LOG-DOSE RESPONSE CURVE FOR SERUM CALCIUM

Vertical Lines Represent the Standard Error of the Mean

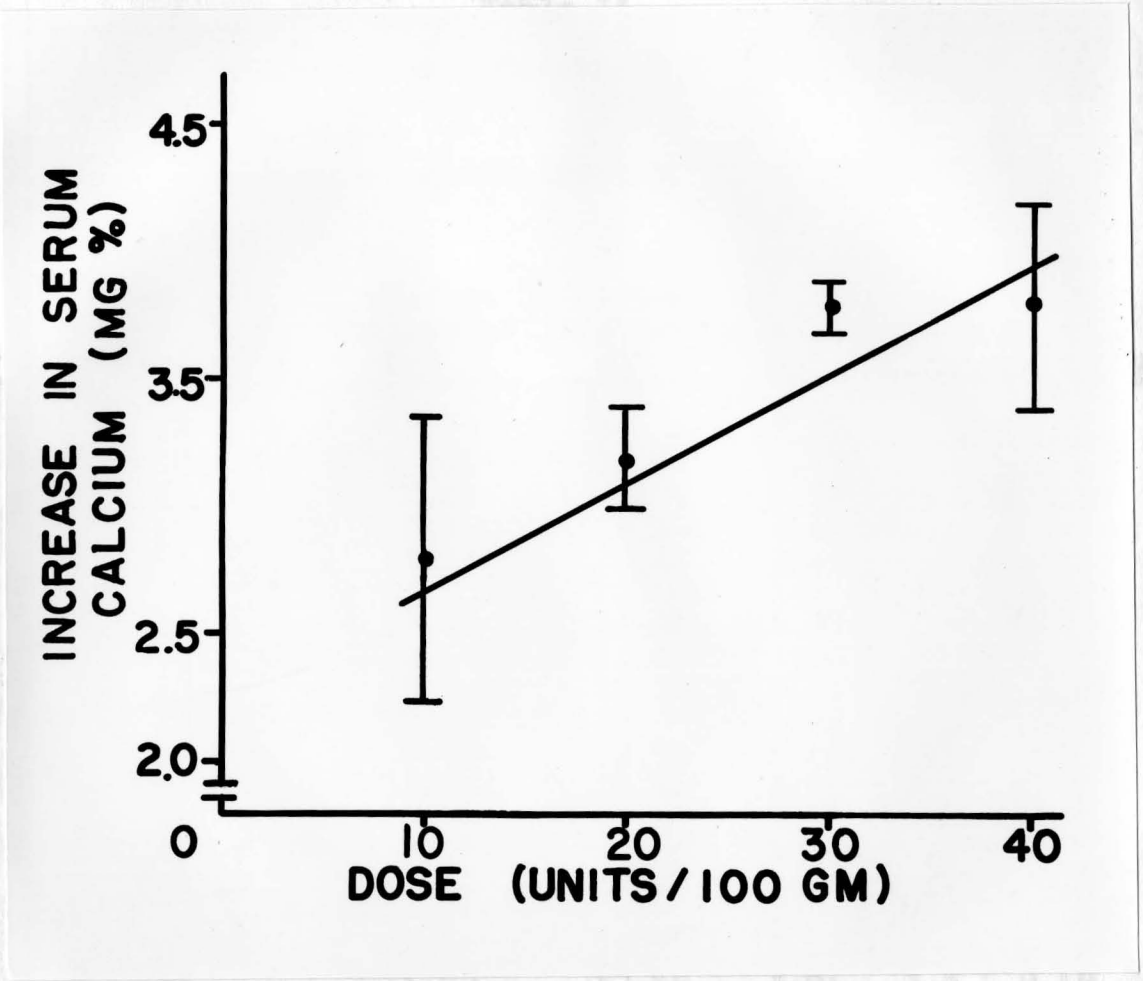

Figure 2

DOSE RESPONSE CURVE FOR ANIMALS ON A NORMAL DIET

The Vertical Lines Represent the Standard Error of the Mean

Table II

DATA FOR THE DOSE RESPONSE CURVE FOR ANIMALS ON A NORMAL DIET

Serum Calcium

Rat #	*Dose (U. S. P. units/100 g)	Before Injection mg%	After Injection mg%	Rise mg%	Mean ± Standard Error
1	10	12.73	13.52	0.79	
2	10	10.02	13.44	3.42	
3	10	7.67	11.59	3.92	2.8 ± 0.56
4	10	7.01	9.44	2.43	
5	10	6.95	10.43	3.48	
6	20	11.09	14.41	3.32	
7	20	10.60	13.60	3.00	
8	20	10.74	13.58	2.84	3.2 ± 0.20
9	20	10.97	15.04	4.07	
10	20	9.32	12.99	3.67	
11	20	10.49	13.30	2.81	
12	20	10.84	13.35	2.51	
13	20	6.68	10.53	3.85	
14	20	7.99	10.39	2.40	
15	30	11.63	15.53	3.90	
16	30	11.80	15.40	3.61	3.8 ± 0.10
17	30	11.61	15.47	3.86	
18	40	11.48	14.60	3.12	
19	40	10.98	14.70	3.72	3.8 ± 0.40
20	40	8.98	13.97	4.98	
21	40	12.72	16.24	3.51	

*Approximate weight of each animal: 150 g.

G. SERUM CALCIUM DETERMINATION

A method of determining ionizable calcium in serum, which is essentially unaffected by organic materials present, has been reported by Ashby and Roberts (2). This method is based upon the fact that at pH's above 12, calcein (an iminoacetate derivative of fluorescein) fluoresces under long-wave ultraviolet light only in the presence of free calcium. In an analysis, a given amount of ethylenediaminetetraacetic acid (EDTA) which is in excess of that required to complex all of the calcium present is added to complex any copper or iron which is present. The solution is back-titrated with standard calcium solution with the calcium replacing the magnesium in any magnesium-EDTA complex which might have formed. When all of the EDTA has become complexed with calcium, additional calcium will combine with calcein causing fluorescence, and the end point will have been reached. Titrations must also be performed on calcium-free samples in order to determine the total calcium binding capacity of the EDTA aliquots. The serum calcium level is determined from the difference between the total calcium binding capacity of the EDTA and the amount of calcium required to titrate the serum sample.

Four hundred microliters of blood sample were obtained from each rat by tail bleeding. The samples were placed in a Beckman/Spinco microfuge and centrifuged for five minutes. Thirty micro-

liter aliquots of serum were added to microtitrator cups. To each cup was added 0.150 ml of 0.001 M EDTA, one drop (about 60 ul) of dilute calcein indicator, one drop of one gm% sodium cyanide solution, and one drop of 1.0 N NaOH. Titration was performed with a microtitrator containing a 20.0 mg% standard calcium solution. A long-wave ultraviolet lamp (Mineralight, Model SL 3660) was placed about 2" above the titration cup. The standard calcium solution was added with continuous stirring until the green fluorescence which was observed no longer increased in intensity. The calcium concentration of the sample is directly proportional to the difference in microliters of standard calcium required to titrate the sample and a water blank. A 10.0 mg% standard calcium solution was run with each group of samples and the calcium concentrations were calculated as follows:

$$\text{mg\% calcium} = 10 \text{ mg\%} \times \frac{\text{microliters for unknown}}{\text{microliters for 10 mg\% standard}}$$

Where:

microliters unknown = microliters of titrant required to titrate blank minus microliters of titrant required to titrate unknown sample.

microliters 10 mg% standard = microliters of titrant required to titrate blank minus microliters of titrant required to titrate 10 mg% standard.

The standard titration curve data appear in Table III. Figure 3 indicates the linear relationship between the microliters of titrant used and the concentration of calcium in solution.

Table III

STANDARD TITRATION CURVE DATA
FOR CALCIUM DETERMINATION

Actual Calcium (mg%)	No. of Samples	Microliters of Titrant ± Standard Deviation	Microliters (Titrant - 0 mg%)
0	5	32.0 ± 1.9	
6	5	21.4 ± 0.37	10.6
8	5	17.7 ± 1.0	14.3
10	6	14.8 ± 0.25	17.2
12	5	12.3 ± 0.26	19.6
14	5	9.7 ± 0.26	22.3
16	5	6.2 ± 0.16	25.8

Solutions:

1. Calcein solution-concentrated: 0.25 g of powdered indicator was dissolved in 4.0 ml of 1.0 N NaOH. When solution was complete, this was diluted to 100 ml with doubly-distilled water.

2. Calcein indicator solution: 0.5 ml of the concentrated calcein solution was diluted to 25 ml with doubly-

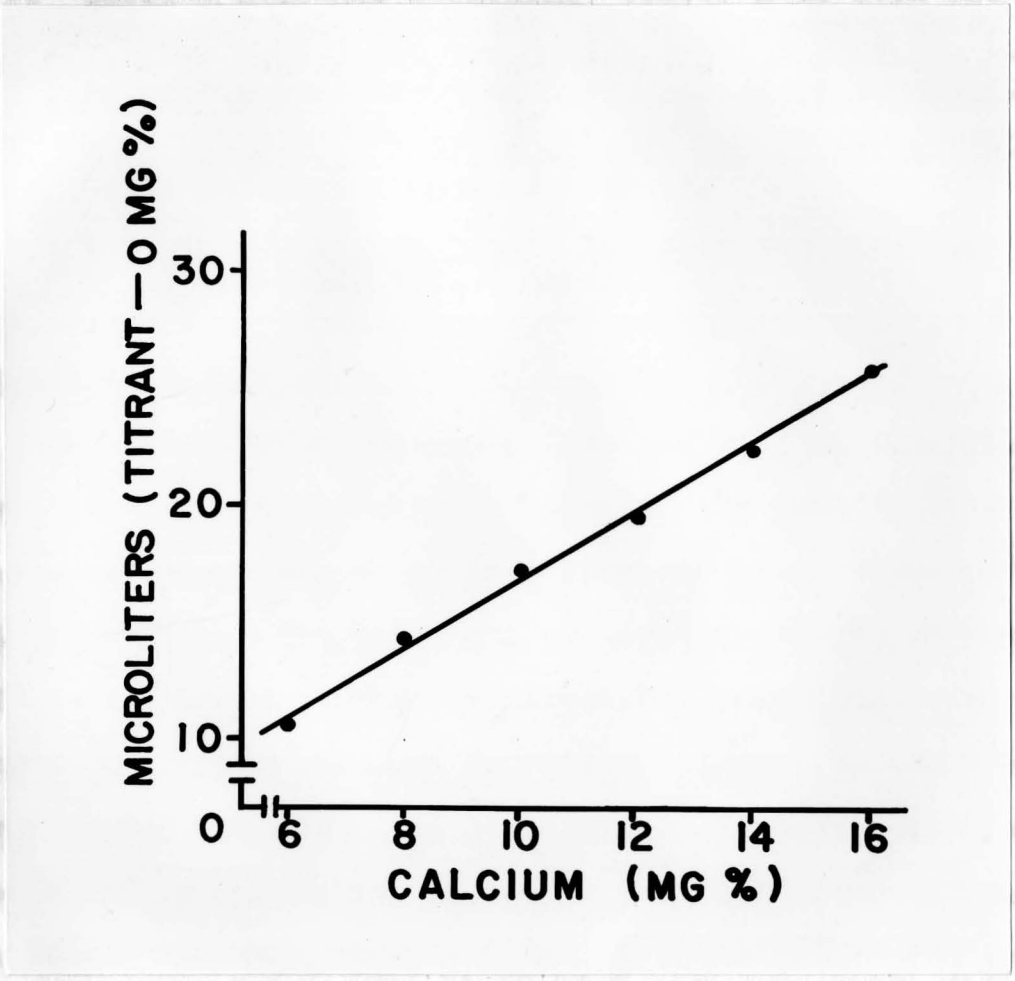

Figure 3

STANDARD CURVE FOR CALCIUM DETERMINATION

distilled water.

3. Calcium standard solution: 0.2497 g of oven-dried calcium carbonate was dissolved in 30 ml of 2 N HCl and diluted to 500 ml with doubly-distilled water. Working solutions were prepared from this stock solution.

4. EDTA standard: 0.375 g of EDTA dissolved in CO_2-free water to total volume of 1000 ml.

5. Sodium cyanide solution: Dissolve one g of NaCN in 100 ml of doubly-distilled water.

H. BLOOD SAMPLING

Blood samples were obtained from the tail of the rat. The rat was placed in a restraining cage and the tail severed approximately three quarters of an inch from the tip. Bleeding was aided by "milking" the tail in a proximal to distal direction. Blood was collected in plastic microtubes, and these were allowed to stand at room temperature for thirty minutes to one hour to allow for clot formation and retraction. Centrifugation was performed in a Beckman/Spinco microfuge for five minutes. Supernatant serum was separated from centrifugate and stored under refrigeration in a microtube until analyzed.

I. THYROPARATHYROIDECTOMY (11)

Male Holtzman albino rats were anesthetized with Nembutal. The dose administered was 35 mg of Nembutal per kg of body weight, and it maintained the rats at a proper level of anesthesia for an hour or longer. The anesthetic takes effect within five minutes

after administration.

After the rat ceased struggling, it was secured on an operating board. The neck of the rat was held taut by a rubber band placed around the upper incisors of the rat and two suitably located nails in the operating board.

An incision about one inch long was made along the midline of the neck. The skin was pulled free from the underlying musculature and the tissue teased apart parallel to the trachea with forceps in order to expose the musculature overlying the trachea. In this procedure, care must be taken not to cut into or bruise the salivary glands, as this will cause profuse bleeding. Forceps were used to tease apart the striated muscle which surrounds the trachea. The musculature of the trachea was retracted with forceps and the trachea exposed.

In the rat, the parathyroid glands are sometimes visible as lighter colored areas at the upper ends of the thyroids. However, they are usually impossible to discern with the naked eye. The entire thyroid-parathyroid apparatus was teased away from the trachea with forceps. The recurrent laryngeal nerve must be visualized and carefully avoided during the dissection. Usually, the two thyroids with connecting isthmus can be removed intact if care is exercised in the operation. It is desirable to remove the organ intact when possible, for this insures completeness of removal and induces less bleeding.

Once the glands are removed, a cotton swab is placed in the area until bleeding ceases. The cotton swab and hemostats are then removed and the wound is closed with two or three stainless steel wound clips. The time for the operation averaged ten minutes.

The animals are returned to the cages for recovery. One day later, a check was made to determine if any of the animals had developed ataxic breathing. Those that had were destroyed.

CHAPTER III

EXPERIMENTAL RESULTS

Experiment 1: Extraction of Parathyroid Hormone from Human Urine with Benzoic Acid

A sample (110 ml) of human urine was collected and to it was added 7.3 ml of concentrated HCl resulting in a pH less than 2. The sample was allowed to remain under refrigeration for a week. This procedure was performed to destroy endogenous hormone (5). To this urine was added 200 U. S. P. units of PTE and the mixture was filtered through Whatman #1 paper in the cold. To the filtrate was added dropwise, over a 30 minute period, 15 ml of a saturated solution of benzoic acid in absolute ethanol. After a six hour stirring period, the suspension was filtered with Whatman #54 paper through a Büchner funnel. The precipitate was then placed in a desiccator under vacuum with sodium hydroxide and silica gel. To the filtrate was added another 10 ml of the saturated benzoic acid solution. This was done in an attempt to assure maximum adsorption of parathyroid hormone by benzoic acid. The stirring was continued for 12 hours after which the precipitate was collected and dried as before.

Three days later, the precipitates were removed and dissolved in 80 ml of absolute ethanol. The solution was centrifuged at 1700 rpm for 30 minutes. The supernatant was discarded and the

precipitate was taken up with 35 ml of absolute ethanol and re-centrifuged at the same speed and time as before. Finally, 13 ml of absolute ethanol were added and the procedure was repeated. The final precipitate was dissolved in 15 ml of ethanol and centrifuged at 8200 rpm for 30 minutes. The precipitate was placed in a desiccator under vacuum with sodium hydroxide and placed in the cold.

On the day of the bioassay, the precipitate was taken up with two drops of 0.2% acetic acid plus 10 ml of 0.9% saline and this was centrifuged for 30 minutes at 8200 rpm. The supernatant was then tested for biological activity.

The animals (140-150 g), maintained on a normal diet, were thyroparathyroidectomized and fasted for 24 hours prior to obtaining the first blood sample. A subcutaneous injection of 3.00 ml of the test material was administered to each animal, and six hours later, the animals were bled again to obtain a second blood sample. The serum calcium level before and after injection was determined.

From Table IV, it is apparent that only a small amount of the added 200 U. S. P. units was recovered from human urine by benzoic acid adsorption.

Table IV

CALCIUM MOBILIZING ACTIVITY OF PARATHYROID HORMONE EXTRACTED FROM HUMAN URINE BY BENZOIC ACID ADSORPTION

Serum Calcium

Rat #	Before Injection mg%	After Injection mg%	Change mg%	Average mg%
1	8.17	9.57	1.40	
2	8.02	8.84	0.82	1.1
3	7.13	7.81	0.68	

Experiment 2: Injection of Human Urine into Rats Containing Parathyroid Hormone

In this experiment, three test media were prepared to see whether or not PTH lost its calcium mobilizing capacity when added to human urine.

To 10 ml of freshly voided human urine was added 300 units of PTE. A second 10 ml sample of urine was acidified to pH 3.0 and to it was added 300 units of PTE. The third test medium was prepared by adding 300 units of PTE to 10 ml of 0.9% saline and acidifying to pH 2.0.

The animals (110-130) were maintained on a normal diet. They were then thyroparathyroidectomized and fasted 24 hours prior to obtaining the first blood sample. Each animal was then given 2.00 ml of the test material subcutaneously and six hours later,

a second blood sample was obtained. The serum calcium before and after injection was determined.

It appears from the data reported in Table V that the hormone is not inactivated when added to human urine.

Table V

CALCIUM MOBILIZING ACTIVITY OF HUMAN URINE CONTAINING PARATHYROID HORMONE

Rat #	Urine + PTE	Acidified Urine + PTE	PTE + Saline	Serum Calcium Change mg%	Average mg%
1	+	-	-	3.81	
2	+	-	-	4.57	4.3
3	+	-	-	4.46	
4	-	+	-	3.17	
5	-	+	-	5.04	3.2
6	-	+	-	1.46	
7	-	-	+	5.13	

Experiment 3: Injection of Filtered Human Urine Containing Parathyroid Hormone into Rats

This experiment was designed to see if filtration of urine by gravity with Whatman #1 paper altered hormonal activity.

Human urine was collected and refrigerated. Twenty-four hours later, the urine was filtered to remove a precipitate which had formed on standing. A sample of 25 ml of urine was then acidified to pH 3.0 with 3 N HCl. To it was now added 500 units of PTE. This was then filtered with Whatman #1 paper. The fil-

trate obtained was the test material.

Animals (120-135 g) were maintained on a normal diet. After their thyroparathyroidectomy, the animals were given distilled water and a calcium deficient diet. Twenty-four hours later, tail blood was obtained and the animals injected with 2.00 ml of the test material subcutaneously. Six hours later, they were bled again to obtain a second blood sample. The serum calcium was measured before and after injection.

The data in Table VI indicates that the calcium mobilizing activity of the hormone was not lost upon filtration with Whatman #1 paper.

Table VI

CALCIUM MOBILIZING ACTIVITY OF FILTERED HUMAN URINE CONTAINING PARATHYROID HORMONE

	Serum Calcium			
Rat #	Before Injection mg%	After Injection mg%	Change mg%	Average mg%
1	10.81	12.11	1.30	
2	6.83	9.74	2.91	2.0
3	9.87	11.58	1.71	

Experiment 4: Extraction of Parathyroid Hormone from Rat Urine with Benzoic Acid

Rats were placed in metabolic cages and placed on powdered

rat chow and tap water. Twelve animals were used and the urine was collected after each 24 hour period and refrigerated. Fresh bags were used to collect each 24 hour urine sample. Urine was collected over a 48 hour period and the final volume of the pooled urine was 187 ml. The urine was then filtered to remove large particles of food and other debris with Whatman #54 filter paper.

To the 187 ml of urine was added 60 units of PTE. This was stirred for a few minutes in the cold room and then acidified to pH 3.5 with 3 N HCl. The urine was filtered in the cold with Whatman #12 paper. To the filtrate was added 19 ml of a saturated solution of benzoic acid in absolute ethanol with constant stirring over a five minute period. After stirring one hour in the cold room, the suspension was filtered through Whatman #54 paper on a Büchner funnel and dried overnight under vacuum in a desiccator with sodium hydroxide and silica gel. The precipitate was then taken up with 200 ml of absolute ethanol and centrifuged at 1000 rpm for 30 minutes in the International Centrifuge at 0°C. The supernatant was discarded and the wash procedure with ethanol was repeated twice. The final precipitate was taken up with 30 ml of ethanol and centrifuged at 8000 rpm for 30 minutes. The supernatant was discarded and the precipitate was transferred to a watch glass and dried overnight under vacuum in a desiccator with sodium hydroxide. Two days later,

the precipitate was mixed with 30 ml of 0.9% saline. This solution was centrifuged at 8000 rpm for 30 minutes. The supernatant was then diluted to 36 ml with saline and this served as the test material.

The animals (160-180 g) were maintained on a calcium deficient diet for three days. On the fourth day, the animals were thyroparathyroidectomized and 12 hours later, were bled to obtain the first blood sample. Doses of 1.00 and 2.00 ml of test material were administered. Six hours later, a second blood sample was taken. The serum calcium before and after injection was determined.

The data in Table VII indicates that none of the original 60 units of PTE were extracted by benzoic acid adsorption.

Table VII

CALCIUM MOBILIZING ACTIVITY OF EXTRACT
OBTAINED FROM RAT URINE
BY BENZOIC ACID ADSORPTION

		Serum Calcium			
Rat #	Dose (ml)	Before Injection mg%	After Injection mg%	Change mg%	Average mg%
1	1.0	14.47	12.93	-1.54	
2	1.0	8.40	7.55	-0.85	
3	1.0	12.23	9.02	-3.21	
4	1.0	9.54	8.70	-0.84	-1.5
5	1.0	9.65	8.23	-1.45	
6	1.0	9.67	8.65	-1.02	

Rat #	Dose (ml)	Before Injection mg%	After Injection mg%	Change mg%	Average mg%
7	2.0	11.04	8.16	-2.88	
8	2.0	10.92	8.74	-2.18	-3.3
9	2.0	13.39	9.24	-4.15	

Experiment 5: Extraction of Parathyroid Hormone from Rat Urine with Benzoic Acid

Twelve animals were placed on powdered rat chow and tap water in metabolic cages. Urine was collected over a two day period at 24 hour intervals and refrigerated. On the third day, the urine was filtered with Whatman #12 filter paper to remove any debris. After filtration, the urine was acidified to pH 3.5 and to it was added 100 units of PTE with constant stirring. The volume of urine was 250 ml. This was centrifuged at 1500 rpm for 30 minutes. The urine was still not clear so it was recentrifuged for another 15 minutes at 2500 rpm. The supernatant was placed in a beaker and to it was added a saturated solution of benzoic acid in absolute ethanol at a rate of four ml every two minutes until 25 ml of the saturated solution had been added. This suspension was stirred for five hours and then filtered with Whatman #54 paper on a Büchner funnel. The precipitate was placed in a desiccator with sodium hydroxide and silica gel and dried under vacuum in the cold overnight. The precipitate was ground up with a mortar and pestle and dissolved in 200 ml of absolute ethanol. The solution was centrifuged for 30 minutes

at 1000 rpm. The supernatant was discarded and the washing was repeated until 700 ml of ethanol had been utilized. The precipitate was transferred to polycarbonate tubes and suspended in 30 ml of ethanol and centrifuged for 30 minutes at 8000 rpm. The precipitate was transferred to a watch glass and placed in a desiccator with sodium hydroxide under vacuum. Prior to the bioassay, the precipitate was dissolved in 30 ml of 0.9% saline and centrifuged at 8000 rpm for 30 minutes. The supernatant was further cleared by centrifugations for another 30 minutes. The second supernatant was the test material.

The animals (160-185 g) used for this experiment had been kept on a normal diet and tap water. After thyroparathyroidectomy, the animals were given two pellets of food. Prior to the bioassay, the animals had been fasted for about 20 hours. Doses of 1, 2, 3, 4 and 5 ml of extract were injected subcutaneously. The animals were returned to their cages and given only tap water. The rats were bled before and six hours after injections. Twelve animals were utilized with each animal acting as its own control. The serum calcium was determined before and after injection of the test material.

As indicated in Table VIII, a definite rise in serum calcium was obtained with a dose equivalent to one-sixth of the total test material but not with smaller doses.

Table VIII

CALCIUM MOBILIZING ACTIVITY OF PARATHYROID HORMONE EXTRACTED FROM RAT URINE BY BENZOIC ACID ADSORPTION

Serum Calcium

Rat #	Dose (ml)	Before Injection mg%	After Injection mg%	Change mg%	Average mg%
1	1.0	8.32	8.07	-0.25	
2	1.0	7.14	7.29	0.15	-1.0
3	1.0	10.35	8.51	-1.84	
4	2.0	8.01	7.78	-0.23	
5	2.0	12.70	11.34	-1.36	-0.5
6	2.0	8.38	8.91	0.53	
7	3.0	6.81	7.67	0.86	
8	3.0	12.97	12.05	-0.92	
9	4.0	10.59	9.17	-1.78	
10	4.0	8.11	7.38	-0.73	
11	5.0	7.29	8.43	1.14	
12	5.0	7.41	10.33	2.92	

Experiment 6: Extraction of Parathyroid Hormone from Rat Urine with Benzoic Acid

Rat urine was collected over a three day period and stored in the cold. The rats were on a normal diet and tap water. The urine was pooled in a beaker and poured out carefully so as not to disturb the debris on the bottom of the vessel. The urine volume was 250 ml and to it was added 100 units of PTE. This was acidified to pH 3.5 and filtered with Whatman #12 paper in the cold. The urine was then allowed to warm up to room tem-

perature before the addition of 25 ml of a saturated solution of benzoic acid. The suspension was placed in the cold and stirred for six hours. The suspension was then filtered with Whatman #54 paper on a Büchner funnel. The precipitate was dried as mentioned in previous experiments. The following day, the precipitate was broken up and dissolved in 200 ml of ethanol and centrifuged at 1600 rpm for 30 minutes. This step was repeated twice using a total of 600 ml of ethanol. The precipitate was removed, placed in a polycarbonate tube with 30 ml of ethanol, and centrifuged at 8000 rpm for 30 minutes. The precipitate was removed and dried as previously mentioned.

Prior to the bioassay, the precipitate was taken up with 30 ml of 0.9% saline and centrifuged at 8000 rpm for 30 minutes. Final volume of test material was 25 ml.

Animals (170-190 g) used in this experiment were maintained on a normal diet. After thyroparathyroidectomy, the animals were given only tap water. Approximately 22 hours later, the assay was started. Animals were bled before and after injection. Doses of 5.0 and 6.0 ml of the test material were administered subcutaneously.

The data in Table IX indicate that a rise in serum calcium was not observed with a dose equivalent to one-fifth and one-fourth of the total test material.

Table IX

CALCIUM MOBILIZING ACTIVITY OF MATERIAL EXTRACTED FROM RAT URINE BY BENZOIC ACID ADSORPTION

		Serum Calcium		
Rat #	Dose (ml)	Before Injection mg%	After Injection mg%	Change mg%
1	5.0	11.51	10.61	-0.90
2	5.0	12.67	11.52	-1.15
3	6.0	8.92	7.93	-0.99
4	6.0	9.32	8.69	-0.63

Experiment 7: Extraction of Parathyroid Hormone from Rat Urine with Benzoic Acid

Urine was collected over a 24 hour period from twelve rats placed in metabolic cages. This was centrifuged for 20 minutes at 1600 rpm to remove debris. The clear urine was acidified to pH 3.2 and to it was added 200 units of PTE. The urine volume was 90 ml, and to this in the cold was added, with constant stirring, 10 ml of saturated solution of benzoic acid in absolute ethanol over a five minute period. Stirring was continued for six hours and the suspension was then filtered and dried as previously mentioned. On the following day, the precipitate was taken up with 150 ml of absolute ethanol and centrifuged for 30 minutes at 1200 rpm. The supernatant was discarded and the procedure was repeated twice. The precipitate was then taken up with 30 ml of absolute ethanol and centrifuged at 8000 rpm for

30 minutes. The resulting precipitate was dried in a desiccator with sodium hydroxide.

Prior to the bioassay, the precipitate was dissolved in 1.5 ml of 0.2% acetic acid and 15 ml of 0.9% saline. This was centrifuged for 30 minutes at 8000 rpm. The supernatant was the test material. The pH of this medium was 4.

The animals (130-145 g) were maintained on a normal diet. After thyroparathyroidectomy, they were fasted for 18 hours before the first blood sample was taken. To each animal was given 2.0 ml of the test material. Six hours later, they were bled again and the serum calcium levels before and after injection were determined.

The data in Table X indicate that no significant rise in serum calcium was obtained with this test material.

Table X

CALCIUM MOBILIZING ACTIVITY OF MATERIAL EXTRACTED FROM RAT URINE BY BENZOIC ACID ADSORPTION

Serum Calcium

Rat #	Before Injection mg%	After Injection mg%	Change mg%	Average mg%
1	9.09	9.60	0.51	
2	11.54	11.15	-0.39	1.0
3	8.60	9.50	0.90	

Experiment 8: Extraction of Parathyroid Hormone from Rat Urine with Benzoic Acid

In this experiment, an excess of the saturated solution of benzoic acid was added during the stirring period in an attempt to assure complete adsorption of PTH.

A sample of human urine (100 ml), which had been refrigerated for 24 hours, was acidified to pH 3.5 with 3 N HCl. To it was added 300 units of PTE and then it was filtered with Whatman #1 paper. To this filtered urine was added 10 ml of the saturated solution of benzoic acid over a period of 15 minutes. The mixture was placed in the cold room and stirred. About two hours later, another 10 ml of the benzoic acid was added. After another two hour period, six more ml of the saturated solution was added. Total time for stirring was six hours after which the suspension was filtered on a Büchner funnel with Whatman #54 paper. The precipitate was broken up and dried in a vacuum desiccator. Two days later, the precipitate was taken up with 150 ml of absolute ethanol and centrifuged at 1500 rpm for 30 minutes. The supernatant was discarded and the procedure was repeated twice. The precipitate was again placed in a vacuum desiccator. On the day of the bioassay, the precipitate was taken up with 20 ml of 0.9% saline and centrifuged at 8000 rpm. The supernatant obtained was the first test medium. To the precipitate was added 10 drops of 0.2% acetic acid and 10 ml of 0.9% saline.

This was also centrifuged at 8000 rpm for 30 minutes. The supernatant obtained here was used as the second injection medium.

The animals (110-120 g) were maintained on a normal diet before thyroparathyroidectomy. They were fasted 20 hours prior to obtaining the first blood sample. A dose of 2.0 ml was administered to each animal. Six hours later, the animals were bled again to obtain the second blood sample.

The data in Table XI show that the addition of an excess of saturated benzoic acid solution did not increase the adsorption of PTH from a urine sample.

Table XI

CALCIUM MOBILIZING ACTIVITY OF MATERIAL EXTRACTED FROM HUMAN URINE BY BENZOIC ACID ADSORPTION

Serum Calcium

Rat #	1st Test Medium	2nd Test Medium	Before Injection mg%	After Injection mg%	Change mg%	Average mg%
1	+	-	7.66	9.19	1.53	
2	+	-	7.87	7.68	-0.19	0.58
3	+	-	8.44	8.04	0.40	
4	-	+	8.83	9.12	-0.29	
5	-	+	9.13	9.69	-0.56	

Experiment 9: Extraction of Parathyroid Hormone from Human Urine with Benzoic Acid

A sample of human urine (115 ml) was collected and refrigerated. Forty-eight hours later, the urine was acidified to

pH 3.5 with 3 N HCl and to it was added 200 units of PTE. The urine was then filtered with Whatman #1 paper and to it, with constant stirring, was added a saturated solution of benzoic acid at a rate of 2 ml/minute until 11 ml of the solution had been added; stirring was continued for six hours. The suspension was filtered with Whatman #54 paper through a Büchner funnel and the precipitate was dried in a vacuum desiccator with sodium hydroxide in the cold. Two days later, the precipitate was taken up with 60 ml of absolute ethanol and centrifuged at 1500 rpm for 30 minutes. This step was repeated twice. The precipitate was transferred to a watch glass and dried in the manner previously mentioned.

Prior to the bioassay, two drops of 0.2% acetic acid and 2 N HCl were added to the precipitate and the mixture was suspended in 11 ml of 0.9% saline. The pH of this medium was less than 2. Two of the animals (145 and 155 g) were each given 2.0 ml of this test material (1st test medium). To the remainder of this test material (about eight ml) was added two more drops of 2 N HCl and 2.5 ml of 0.9% saline. Two animals (160 g) were each given 2.0 ml of this test material (2nd test medium); one dose was administered subcutaneously and the other intraperitoneally.

The animals were maintained on a normal diet prior to the bioassay. They were thyroparathyroidectomized and bled 24 hours later. The injections were made and the animals were bled again

six hours later. The serum calcium was determined before and after injection, and the results appear in Table XII. It appears that the addition of further acid to the test medium resulted in the further dissolution of the active principle.

Table XII

CALCIUM MOBILIZING ACTIVITY OF MATERIAL EXTRACTED FROM HUMAN URINE BY BENZOIC ACID ADSORPTION

Serum Calcium

Rat #	1st Test Medium	2nd Test Medium	Before Injection mg%	After Injection mg%	Change mg%
1	+	-	6.60	7.19	0.59
2	+	-	7.65	7.51	-0.14
3	-	+	11.04	12.26	1.22
*4	-	+	9.89	11.75	1.86

*Test material injected intraperitoneally.

Experiment 10: Extraction of a Hypercalcemic Principle from Urine Collected from Hyperparathyroid Rats

Three normal rats (two females and one male) were given 200 units of PTE subcutaneously while under ether anesthesia. They were then placed in metabolic cages and given food and water. Under each cage was placed a urine collection bag (Whirl-pak bag, Scientific Products). The bags were collected 24 hours later and the urine was refrigerated. Fresh bags were placed under each cage and the injection procedure was repeated with each animal

receiving another 200 units of PTE. The two day collection of urine was centrifuged at 1000 rpm for 10 minutes to remove debris and then lyophilized. The precipitate was dissolved in a few drops of 0.2% acetic acid and placed in a freezer. Before use, the sample was allowed to thaw before it was taken up with 0.9% saline to make the final volume of the medium approximately 25 ml. This solution was centrifuged at 2000 rpm for five minutes. The supernatant was acidified to pH 4.0 (initial pH was 7.5) and this was the first test medium. The precipitate obtained from the centrifugation was taken up with a few drops of 0.2% acetic acid and 10 ml of 0.9% saline. The precipitate was still insoluble so a few drops of 3 N HCl were added and this caused much of the precipitate to go into solution. The final pH of the medium was less than 2 and this was the second test medium.

The animals (130-145 g) were maintained on a normal diet. After thyroparathyroidectomy, they were given distilled water and a calcium deficient diet. Twenty-four hours later, the first blood sample was taken and the animals were injected with 3.0 ml of a particular test medium. Six hours later, they were bled again and the serum calcium levels before and after injection were determined. One animal died in the first test medium group shortly after injection.

It is apparent from the data in Table XIII that no hyper-

calcemic principle was extracted from the urine of hyperparathyroid rats under the conditions of the experiment.

Table XIII

CALCIUM MOBILIZING ACTIVITY OF MATERIAL EXTRACTED FROM THE URINE OF HYPERPARATHYROID RATS

Serum Calcium

Rat #	1st Test Medium	2nd Test Medium	Before Injection mg%	After Injection mg%	Change mg%	Average mg%
1	-	+	12.28	9.82	-2.46	
2	-	+	11.64	8.76	-2.88	-2.3
3	-	+	11.73	10.26	-1.47	
4	+	-	13.69	11.09	-2.60	
5	+	-	11.25	10.23	-1.02	

Experiment 11: Extraction of Parathyroid Hormone from Human Urine with Benzoic Acid and Cysteine-HCl

In this experiment, cysteine-HCl was utilized in an attempt to prevent the possible oxidation by air of PTH during the recovery procedure.

A sample of human urine was refrigerated immediately after voiding. Twenty-four hours later, the urine was filtered with Whatman #1 paper. A sample of 100 ml of this urine was acidified to pH 3.0 and to it was added 20 ml of 0.125 M cysteine-HCl in 0.9% sodium chloride solution and 500 U. S. P. units of PTE (5.0 ml).

This solution was taken to the cold room and to it was added, with constant stirring, 10 ml of a saturated solution of benzoic

acid in absolute ethanol at a rate of one ml every three minutes until all the solution was added. After stirring for six hours, the suspension was filtered on a Büchner funnel with Whatman #54 paper. The precipitate was placed in a desiccator with sodium hydroxide under vacuum and placed in the cold.

On the following day, the precipitate was taken up with 200 ml of absolute ethanol and centrifuged at 2500 rpm for 30 minutes. The centrifugation procedure was repeated twice. The final precipitate was placed in a 50 ml polycarbonate tube, taken up with 30 ml of ethanol and centrifuged at 8200 rpm for 30 minutes. The precipitate was again dried in a desiccator as previously mentioned.

On the day of the bioassay, 10 drops of 0.2% acetic acid plus 10 ml of 0.9% saline were added to the precipitate. It was centrifuged at 8200 rpm for 30 minutes. The supernatant was the test material.

The animals (150-160 g) were maintained on a calcium deficient diet and distilled water for three days. They were thyroparathyroidectomized and 16 hours later, the first blood samples were drawn. Each animal then received 2.00 ml of the test material subcutaneously. Six hours later, they were bled again and the serum calcium before and after injection was determined.

The data in Table XIV indicate that addition of cysteine-HCl to urine may enhance the recovery of PTH from urine samples.

Table XIV

CALCIUM MOBILIZING ACTIVITY OF MATERIAL EXTRACTED FROM HUMAN URINE BY BENZOIC ACID ADSORPTION

	Serum Calcium			
Rat #	Before Injection mg%	After Injection mg%	Change mg%	Average mg%
1	11.03	13.81	2.78	
2	12.23	12.14	-0.09	1.6
3	10.90	13.11	2.21	

Experiment 12: Extraction with Benzoic Acid of a Hypercalcemic Principle from the Urine of Hyperparathyroid Rats

In the last experiment, a significant rise in serum calcium was observed with test media obtained from urine samples to which had been added cysteine hydrochloride to prevent air oxidation of exogenous parathyroid hormone. With this in mind, cysteine hydrochloride was added to the plastic bags used for the collection of urine from hyperparathyroid rats.

Four rats were placed in metabolic cages after each had been given 300 units of PTE subcutaneously. In each of the urine collecting bags was placed 1 ml of 0.125 M cysteine-HCl. Twenty-four hours later the urine was collected, centrifuged at 1200 rpm for 10 minutes to remove debris, and then acidified to pH 3.0. The solution was placed in the cold and to it was added five ml of 0.125 M cysteine-HCl. The rats were again injected with 300

units of PTE and placed in the metabolic cages. This time, 2.0 ml of cysteine-HCl plus 2.0 ml of 3 N HCl were placed in the urine collecting bags. Twenty-four hours later, the urine was collected and acidified as previously mentioned.

The two urine collections were pooled to give a final sample of 100 ml. To it was added 15 ml of 0.25 M cysteine-HCl and 11 ml of a saturated solution of benzoic acid in absolute ethanol. This medium was stirred for seven hours in the cold and then filtered on a Büchner funnel with Whatman #54 paper. The precipitate was dried in a vacuum desiccator in the cold.

On the following day, the precipitate was taken up with 100 ml of absolute ethanol and centrifuged at 1200 rpm for 30 minutes. This procedure was repeated twice. The precipitate was taken up with another 30 ml of ethanol and centrifuged at 8200 rpm for 30 minutes. The collected precipitate was dried again in a vacuum desiccator.

On the day of the bioassay, the precipitate was taken up with 10 drops of 0.2% acetic acid plus 10 ml of 0.9% saline and centrifuged at 8200 rpm for 30 minutes. The supernatant was the test extract. Each animal received 2.0 ml of the test material.

The animals (120 g) were maintained on a normal diet. They were thyroparathyroidectomized and 24 hours later, the first serum samples were drawn. Each animal was then injected subcutaneously and six hours later, bled again to obtain a second

serum sample. The serum calcium before and after injection was determined. Unexpectedly, the data in Table XV did not corroborate previous evidence that use of cysteine hydrochloride favored recovery of the active principle from urine.

Table XV

CALCIUM MOBILIZING ACTIVITY OF MATERIAL EXTRACTED FROM THE URINE OF HYPERPARATHYROID RATS

	Serum Calcium			
Rat #	Before Injection mg%	After Injection mg%	Change mg%	Average mg%
1	8.06	8.25	0.19	-0.41
2	8.99	7.63	-1.36	
3	7.68	7.48	-0.20	
4	7.96	7.70	-0.26	

Experiment 13: Extraction of Parathyroid Hormone from Rat Urine Using Silica Gel Particles

Having been unsuccessful with the benzoic acid extraction procedure, an attempt was made to utilize silica gel to extract parathyroid hormone (14).

Twelve animals were placed in metabolic cages and given rat chow and tap water. Urine was collected over a 16 hour period and refrigerated. It was then centrifuged at 1800 rpm for 10 minutes to remove any debris. After centrifugation, 60 ml of

urine was poured off and to it was added 200 units of PTE. The urine was acidified to pH 3.5. It was filtered with Whatman #1 paper by gravity in the cold and to it was added 500 mg of silica gel ("Quso G32", Philadelphia Quartz Company, Philadelphia), with constant stirring, over a five minute period at room temperature. The suspension of urine and silica was centrifuged at 2000 rpm for 30 minutes. The supernatant was discarded and to each tube was added 30 ml of doubly-distilled water. This was mixed thoroughly and centrifuged at 2000 rpm for 30 minutes. Again, the supernatant was discarded and the precipitate was taken up with 25 ml of 20% acetone in 1% acetic acid and centrifuged for 30 minutes at 2000 rpm. The acetone-acetic acid mixture was used to elute PTH from the silica gel particles.

The final supernatant was poured into a beaker and the silica gel was discarded. Most of the acetone was removed from the solution before lyophilization. The precipitate obtained after lyophilization was taken up with about 2.0 ml of 0.2% acetic acid and 15 ml of 0.9% saline. The mixture was centrifuged at 8000 rpm for 30 minutes to remove insoluble constituents. The supernatant (about 15 ml) was the test material. It exhibited a pH of 4.

The animals (120-130 g), maintained on a normal rat chow diet, were thyroparathyroidectomized. They were then returned to their cages and given only tap water. Eighteen hours later, the

animals were bled from the tail and 2.0 ml of the test material was injected subcutaneously. Six hours later, they were bled again and serum calcium levels before and after injection were determined.

The data in Table XVI indicate that silica gel did not appear useful in the recovery of parathyroid hormone from urine.

Table XVI

CALCIUM MOBILIZING ACTIVITY OF MATERIAL EXTRACTED FROM HUMAN URINE WITH SILICA GEL

	Serum Calcium			
Rat #	Before Injection mg%	After Injection mg%	Change mg%	Average mg%
1	7.59	7.07	-0.52	
2	9.56	9.27	-0.29	0.35
3	7.18	8.34	1.16	

Experiment 14: Extraction of Parathyroid Hormone from Human Urine Using Silica Gel

In the last experiment, addition of silica gel was followed by a five minute stirring period. In this experiment, a five hour stirring period was attempted in the hope of increasing the recovery of PTH.

Freshly voided human urine (80 ml) was collected and refrigerated. Three hours later, the urine was acidified to pH

3.3 and to it was added 200 units of PTE with constant stirring for two minutes at room temperature. The urine was then filtered with Whatman #1 paper in the cold. After filtration, 500 mg of Quso G32 was added and this was stirred for five hours in the cold. The urine sample was placed under refrigeration. Two days later, the mixture was centrifuged at 2600 rpm for 15 minutes. The supernatant was discarded and the precipitate was taken up with 80 ml of doubly-distilled water. This was centrifuged at 2700 rpm for 30 minutes. The supernatant was again discarded and the precipitate was dissolved with 20 ml of a 1% acetone-5% acetic acid solution. This was centrifuged at 3000 rpm for 30 minutes and the supernatant was lyophilized. The dry material was taken up with two drops of 0.2% acetic acid and 10 ml of 0.9% saline and centrifuged at 8000 rpm for 30 minutes to remove insoluble components. The supernatant was the test material (about 10 ml).

The animals (120-130 g) were maintained on a normal diet before thyroparathyroidectomy. Twenty-four hours later, the assay was started. Subcutaneous injections of 2.0 ml of the test material were administered. Six hours later, the animals were bled again.

The data in Table XVII indicate again that the use of silica gel was not effective in the recovery of exogenous hormone from urine.

Table XVII

CALCIUM MOBILIZING ACTIVITY OF MATERIAL EXTRACTED FROM HUMAN URINE WITH SILICA GEL

Serum Calcium

Rat #	Before Injection mg%	After Injection mg%	Change mg%	Average mg%
1	7.33	6.59	-0.74	
2	7.60	6.88	-0.72	-0.64
3	6.51	6.69	0.18	

Experiment 15: Concentration of Parathyroid Hormone from Human Urine Using Sephadex G-25

Since silica gel, like benzoic acid, did not prove to be effective in extracting PTH from urine, an attempt was now made to utilize Sephadex in extracting the hormone.

Normal human urine (75 ml) was collected and refrigerated. Twenty-four hours later, the urine was acidified to pH 3.3 with 3 N HCl. To the urine was added 200 units of PTE; this was stirred for 15 minutes in the cold. The urine was then filtered with Whatman #1 paper in the cold and to the filtrate was added 30 g of Sephadex G-25 coarse beads (water regain, 2.5 ml water/g of dry Sephadex). The solution was left in the cold for 30 minutes to allow for sufficient swelling of Sephadex particles. The Sephadex was then packed into Filtefuge tubes and centrifuged at 2000 rpm for 10 minutes. The urine was collected in serum

bottles and lyophilized. To the precipitate was added 10 drops of 0.2% acetic acid and 10 ml of 0.9% saline. This was the test material.

The animals (120-130 g) were maintained on a normal diet and tap water. After thyroparathyroidectomy, they were given one pellet of food. Twenty-four hours later, the first blood sample was taken. Each animal was then given 2.0 ml of the test material. Six hours later, the animals were bled again to obtain a second blood sample. The serum calcium before and after injection was determined.

The data in Table XVIII indicate that PTH was not extracted from human urine with Sephadex G-25.

Table XVIII

CALCIUM MOBILIZING ACTIVITY OF MATERIAL EXTRACTED FROM HUMAN URINE WITH SEPHADEX G-25

	Serum Calcium			
Rat #	Before Injection mg%	After Injection mg%	Change mg%	Average mg%
1	11.98	11.49	-0.49	
2	7.77	6.98	-0.88	-0.96
3	8.33	6.83	-1.53	

Experiment 16: Concentration of Parathyroid Hormone from Human Urine Using Sephadex G-25

Human urine (115 ml) was collected and refrigerated. On the

following day, the urine was acidified to pH 3.4. To this was added 200 units of PTE; the sample was stirred for 10 minutes in the cold. The urine was filtered with Whatman #1 paper in the cold and to it was added 35 g of Sephadex G-25 (coarse beads). The Sephadex was allowed to swell for 60 minutes in the cold. After swelling, it was placed in Filtefuge tubes and centrifuged at 2000 rpm for 10 minutes. The remaining urine, about 25 ml, was poured into a one liter beaker and to it was added another 10 g of Sephadex. This was allowed to swell for 20 minutes; it was then centrifuged at 2000 rpm for 20 minutes. The collected urine was lyophilized. To the precipitate was added a few drops of 0.2% acetic acid and 10 ml of 0.9% saline. The solution was centrifuged at 8000 rpm for 30 minutes and the supernatant was used as the test material.

The animals (120-150 g) were maintained on a normal diet. After thyroparathyroidectomy, they were given one pellet of food. Twenty-four hours later, the first blood sample was taken and 3.0 ml of the test material was injected. Six hours later, the animals were bled again. Serum calcium levels were determined.

The data in Table XIX show again that Sephadex G-25 did not extract parathyroid hormone from urine under the conditions specified.

Table XIX

CALCIUM MOBILIZING ACTIVITY OF MATERIAL EXTRACTED FROM HUMAN URINE USING SEPHADEX G-25

	Serum Calcium			
Rat #	Before Injection mg%	After Injection mg%	Change mg%	Average mg%
1	12.16	11.35	-0.81	
2	7.72	7.05	-0.67	-0.74
3	8.40	8.17	-0.23	

Experiment 17: Concentration of Parathyroid Hormone from Human Urine Using Sephadex G-25

In this experiment, an attempt was also made to elute any parathyroid hormone that may have been adsorbed by the Sephadex particles.

A sample of human urine (150 ml), which had been refrigerated for 20 hours, was filtered by gravity with Whatman #54 paper. The urine was acidified to pH 3.5 and to it was added 300 units of PTE. The urine developed a cloudy appearance upon addition of PTE but it was not filtered again. To it was added 35 g of Sephadex with constant stirring; the medium was placed in the cold for 60 minutes. It was then placed in Filtefuge tubes and centrifuged at 1500 rpm for 10 minutes. The urine obtained was lyophilized. The precipitate was taken up with 20 ml of 0.9% saline and this was the first test medium.

To the Sephadex remaining after centrifugation at 1500 rpm for 10 minutes, was added 90 ml of 1 x 10^{-3} M saline. This mixture was centrifuged at 2000 rpm for 10 minutes. The washed sample was lyophilized. To the precipitate was added 20 ml of 0.9% saline and this served as the second test medium.

Animals (110-130 g) that were maintained on a normal diet, were thyroparathyroidectomized and given only tap water. Twenty-four hours later, they were bled to obtain the first blood sample. Each animal was given 2.0 ml of test material and six hours later they were bled again. The injection of the mediums resulted in a traumatic experience for all animals, even to the extent of causing death of one of the animals in the second test medium group.

Equivocal results were obtained in this experiment as is indicated from the data in Table XX.

Table XX

CALCIUM MOBILIZING ACTIVITY OF MATERIAL EXTRACTED FROM HUMAN URINE USING SEPHADEX G-25

Serum Calcium

Rat #	1st Test Medium	2nd Test Medium	Before Injection mg%	After Injection mg%	Change mg%	Average mg%
1	+	-	9.17	9.31	0.14	
2	+	-	8.32	8.88	0.56	1.4
3	+	-	9.09	12.55	3.46	

Rat #	1st Test Medium	2nd Test Medium	Before Injection mg%	After Injection mg%	Change mg%	Average mg%
4	-	+	8.78	7.32	1.46	
5	-	+	7.84	7.51	-0.33	

Experiment 18: Use of Sephadex to Concentrate Parathyroid Extract

This experiment was designed to test whether or not two different grades of Sephadex, i. e. G-10 and G-25, could be utilized to profitably concentrate parathyroid hormone activity.

To 5.0 ml (500 units) of PTE was added 1.0 g of G-25. To another 5.0 ml of PTE was added 2.0 g of G-10. After addition, the samples were placed in the cold for 45 minutes. The mixtures were then placed in Filtefuge tubes and centrifuged at 2000 rpm for 20 minutes. The fraction collected from each sample was placed in a serum bottle. In the case of G-25, there was a noticeable precipitate remaining in the Filtefuge tube. This precipitate was retained.

The two collected samples (from G-10 and G-25) were lyophilized in the serum bottles. Three ml of PTE was also lyophilized as a control. To the dry material in each bottle was added 10 drops of 0.2% acetic acid plus 10 ml of 0.9% saline. These same reagents were added to the previously mentioned precipitate remaining after G-25 centrifugation. The test volume in each case was about 10 ml and each animal was given 2.0 ml subcuta-

neously.

The animals (150-180 g) were maintained on a calcium deficient diet and distilled water for three days. They were thyroparathyroidectomized and 16 hours later, were bled to obtain the first serum samples. The injections were made and the animals were bled six hours later. Serum calcium was determined before and after injection.

The data in Table XXI show that two different grades of Sephadex were able to concentrate parathyroid hormone in commercial extract preparations. G-10 appears to be much more effective than G-25 in this approach. The formation of a noticeable precipitate in the Filtefuge tube where G-25 was used was unexpected and is unexplainable at this time. Since this precipitate showed appreciable biological activity, it appears that Sephadex G-10 would be the better choice for further studies on the concentration of the hormone from urine.

Table XXI

CALCIUM MOBILIZING ACTIVITY OF PARATHYROID HORMONE CONCENTRATED FROM PTE WITH SEPHADEX G-10 AND G-25

Serum Calcium

Test Medium	Rat #	Before Injection mg%	After Injection mg%	Change mg%	Average mg%
G-10	1	10.41	17.43	7.02	
	2	11.60	17.60	6.00	5.8
	3	13.25	17.72	4.47	
G-25	4	13.09	12.88	-0.21	
	5	12.26	13.92	1.66	1.5
	6	11.59	14.69	3.10	
G-25 (Precipitate)	7	11.43	15.65	4.22	
	8	12.23	15.23	3.00	3.6
	9	9.34	13.04	3.70	
PTE (300 U. S. P. units lyophilized)	10	11.51	13.30	1.79	
	11	11.04	12.86	1.82	2.1
	12	10.91	13.58	2.67	

CHAPTER IV

DISCUSSION AND CONCLUSIONS

Many attempts and various methods were employed to extract or concentrate parathyroid hormone contained in both human and rat urine. The method of estimation of biological activity of the test material was based upon a rise in serum calcium, six hours after subcutaneous injection of the test material into thyroparathyroidectomized animals. In establishing the standard dose response curves, a mean rise in serum calcium of 3.8 mg% was obtained after injection of 15 U. S. P. units of PTE into animals maintained on a calcium-deficient diet whereas a rise of 2.8 mg% was observed when a dose of 10 U. S. P. units/100 grams of body weight was given to animals maintained on a normal diet. In either case, the method of estimating the hypercalcemic response was sensitive to a relatively low concentration of PTH and thus appeared to be satisfactory for the work under consideration.

In the benzoic acid adsorption experiments, various modifications were made in an attempt to recover exogenous hormone from urine. Dissolution of the final precipitate with 0.2% acetic acid and/or 2 N HCl as well as 0.9% saline was employed. An excessive amount of benzoic acid was added to the urine containing the hormone in an attempt to assure maximum adsorption of the

hormone. Neither of these modifications appeared to enhance the recovery of the hormone by the method of Eliel et al. (7) applied here.

It was suspected that the hormone may have been inactivated by oxygen during the six hour stirring period used in the benzoic acid extraction. To eliminate this possibility, cysteine-HCl was added to a volume of urine containing 500 U. S. P. units of PTH. The final volume of the test medium after extraction with benzoic acid was 10 ml. An injection of 2.0 ml of this test solution into a group of animals maintained on the normal diet, was found to raise the serum calcium by 1.6 mg%. From the standard dose response curve for animals on the same diet, this increase would correspond to a dose of less than 15 U. S. P. units. However, test material obtained under conditions in which cysteine-HCl was omitted in the extraction procedure had a similar biological activity, and hence use of this adjunct was not useful under the conditions employed here for the extraction of PTH from urine.

The use of silica gel particles for extraction of PTH, as had been applied by Rosselin et al. (14), proved to be as ineffective as the use of benzoic acid in the extraction of PTH from urine.

In another approach, Sephadex G-25 was utilized in an attempt to concentrate a specified volume of urine containing PTH.

Again, the rise in serum calcium obtained with the test material was below the lowest values (2.8 mg% and 3.8 mg%) obtained in establishing the dose response curves. Sephadex G-25 coarse beads have an estimated fractionation range of molecular weight of 5,000. This means that molecules which have a molecular weight of 5,000 or more will be excluded and will exist in the interstitial spaces of the Sephadex which has been added to the particular test solution. Therefore, PTH with an estimated molecular weight of 8,500 should be readily excluded and recovered upon centrifugation in the Filtefuge tubes used in these experiments. However, the fractionation ranges are approximate, because fractionation depends not only on the size of the molecules to be separated, but also on their shape and their chemical structure. Therefore, exact data on the ranges in which good separation can be achieved are difficult to ascertain. It may be that PTH in an acidic medium exhibits a chemical structure conducive for entry into the gel particles of G-25. This led to the investigation of G-10, a grade of Sephadex with a lower fractionation range.

When G-10, which has a fractionation range of molecular weight of 700, and G-25 were added separately to known volumes of PTE, it was found that G-10 was more effective in concentrating the volume of PTE such that the remaining solution contained a higher concentration of hormonal activity. The test material ob-

tained in the Filtefuge tubes with the use of G-10 caused an increase of 5.8 mg% in the serum calcium level when injected into thyroparathyroidectomized animals. On the other hand, a serum calcium increase of 1.5 mg% was reported for material excluded by G-25.

With regard to the adsorption phenomenon, Sephadex particles can adsorb basic amino acids because of an equivalent amount of carboxyl groups on the gel matrix. Elution with a salt solution apparently eliminates this interaction of basic amino acids and the carboxyl groups. In an attempt to investigate this possible interaction as a contributing factor in the retention of PTH molecules, G-25 was washed with 1×10^{-3} M saline after it had been used to concentrate a volume of urine containing a specified amount of PTH. It was found that this washing did not enhance the recovery of PTH, indicating that the retention of PTH molecules by G-25 was not responsible for the low biological activity of the excluded material.

The results previously reported utilizing Sephadex G-10 and G-25 show that G-10 is more effective in excluding PTH molecules. This suggests that further investigation be done with this grade of Sephadex in studies on the recovery of parathyroid hormone from urine.

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ABSTRACT

Abstract of the thesis entitled "STUDIES ON THE EXTRACTION OF PARATHYROID HORMONE FROM URINE" submitted by Rodger S. Izzo in partial fulfillment of the requirements for the degree of Master of Science, June 1967.

Benzoic acid, silica gel particles and Sephadex G-25 were used in an attempt to extract or concentrate parathyroid hormone contained in both rat and human urine. The method of estimation of biological activity of the test material was based upon a rise in serum calcium, six hours after subcutaneous injection of the test material into thyroparathyroidectomized rats.

The use of benzoic acid to extract PTH from urine did not prove to be effective. In attempts to alter the benzoic acid extraction procedure, such as dissolution of the final precipitate with acidified saline instead of saline alone, and addition of an excessive amount of benzoic acid to assure maximum adsorption of hormone, it was found that the recovery of the hormone was not enhanced.

The use of silica gel particles was not effective in extracting hormonal activity from either human or rat urine.

When Sephadex G-25 was used to concentrate PTH in urine samples, the biological activity of the excluded molecules was too low to warrant further use of this gel matrix. Investigation

of G-10 showed that this grade of Sephadex was more effective in excluding PTH molecules than was G-25 and should prove useful in further investigations on the recovery of parathyroid hormone from urine.

APPROVAL SHEET

The thesis submitted by Rodger Samuel Izzo has been read and approved by the undersigned member of the faculty of the Loyola University Stritch School of Medicine who served as director of the research program.

The final copies have also been examined and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

May 29, 1967
Date

Maurice J. LiVecchi
Signature of Adviser